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(56) Documents cited
GB 2116997 A GB 1383216 A EP 0056111 A1
EP 0052829 A2 WO 80/02033 A1 JP 63105678 A
US 4518693 A
Ger. Chem. Eng. 1984, 7(4), 233-240
Biotechnol. Ser. 1985, 5, 71-92

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UK CL (Edition K) C3H HH1, C6F FF
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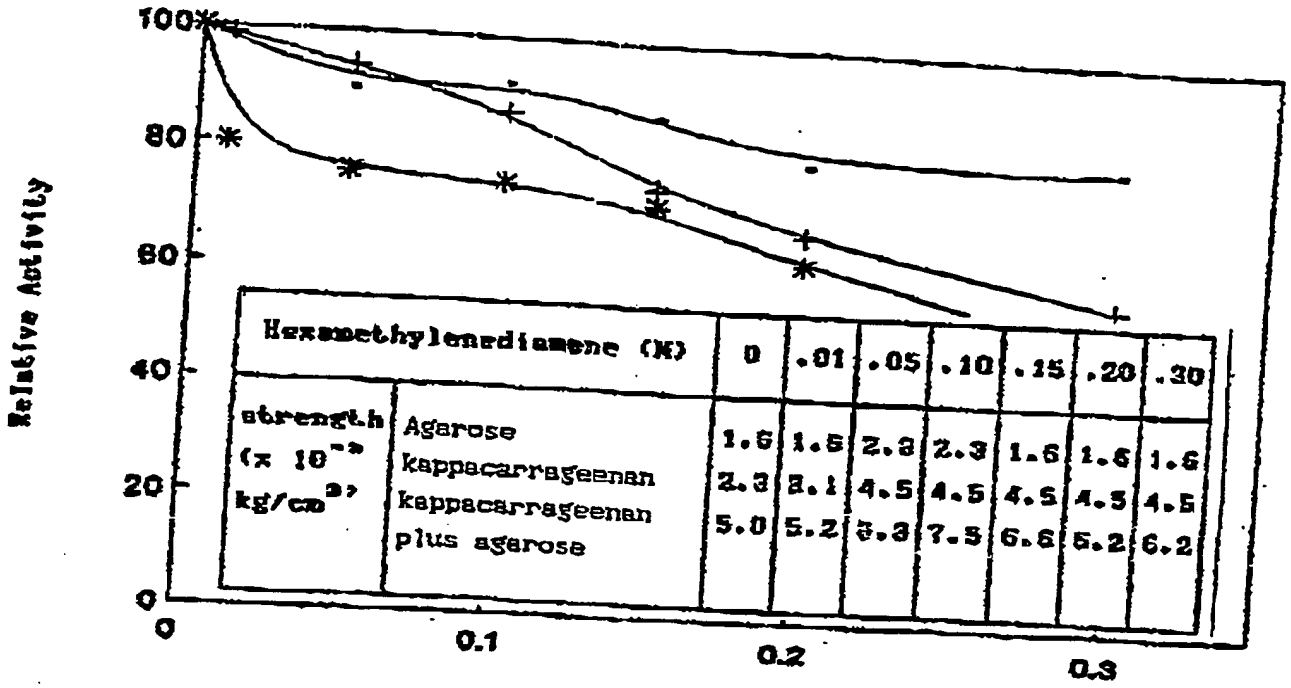
Online databases: WPI, DIALOG/BIOTECH

(54) Gel immobilised enzymatically-active material

(57) Enzymatically-active material, including whole cells, is immobilised in a gel matrix of agarose and/or carrageenan, which may be cross-linked. The material may be penicillin acylase for the deacylation of penicillin G.

GB 2244711 A



Hexamethylenediamene (M)		0	.01	.05	.10	.15	.20	.30
strength ($\times 10^{-2}$ kg/cm^2)	Agarose	1.6	1.6	2.3	2.3	1.6	1.6	1.6
	kappacarrageenan	2.3	3.1	4.5	4.5	4.5	4.5	4.5
	kappacarrageenan plus agarose	5.0	5.2	6.3	7.5	6.6	6.2	6.2

Fig. 1

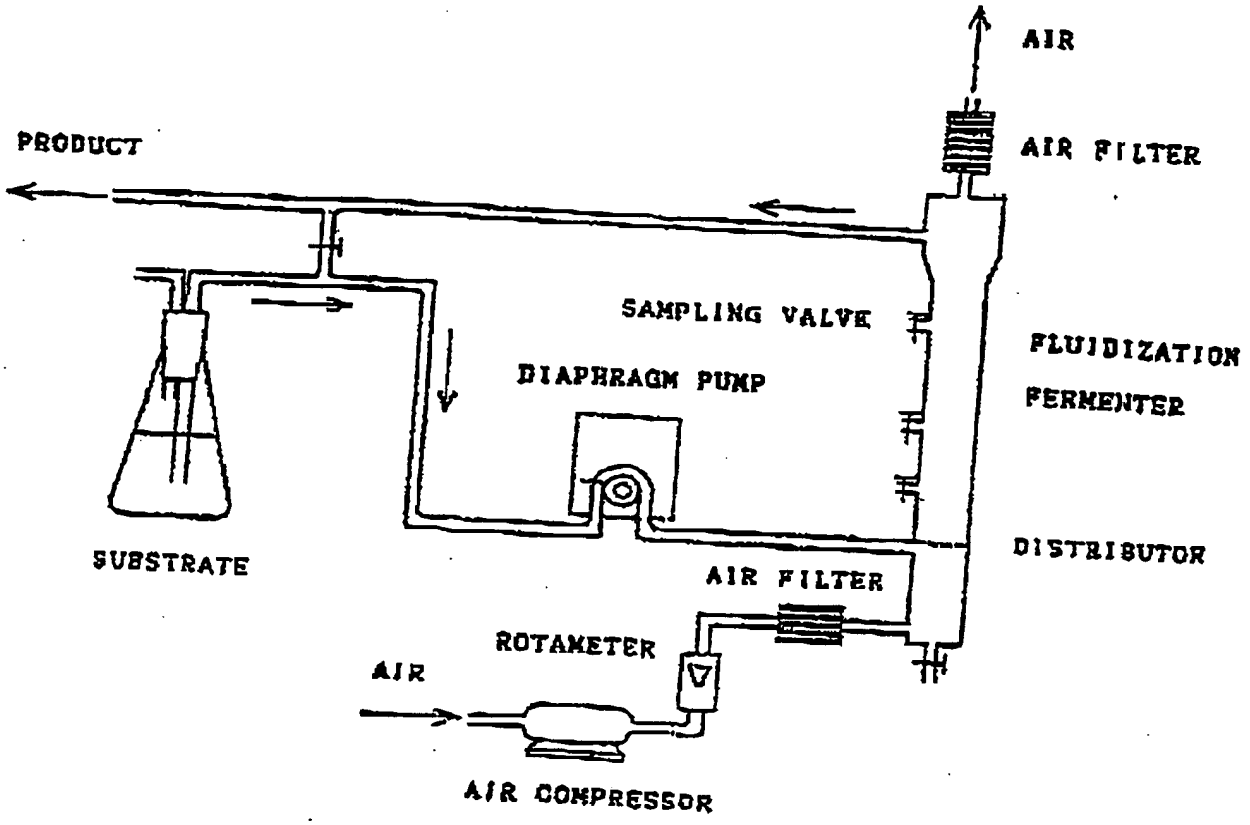


Fig 2 Details and Diagram of Fluidization Fermenter System

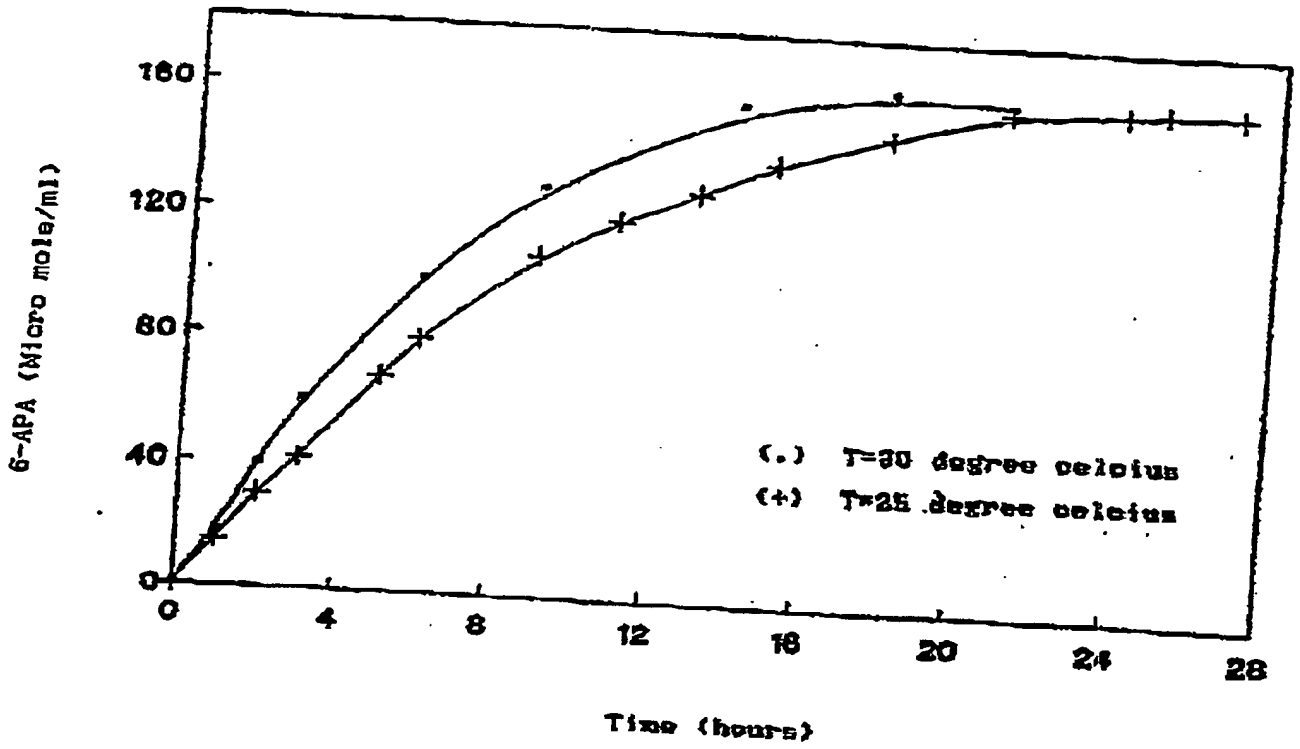

6-APA (Micro mole/ml)
160
120
80
40
0
0
4
8
12
16
20
24
28
Time (hours)
(.) T=30 degree celcius
(+) T=25 degree celcius

Fig. 3

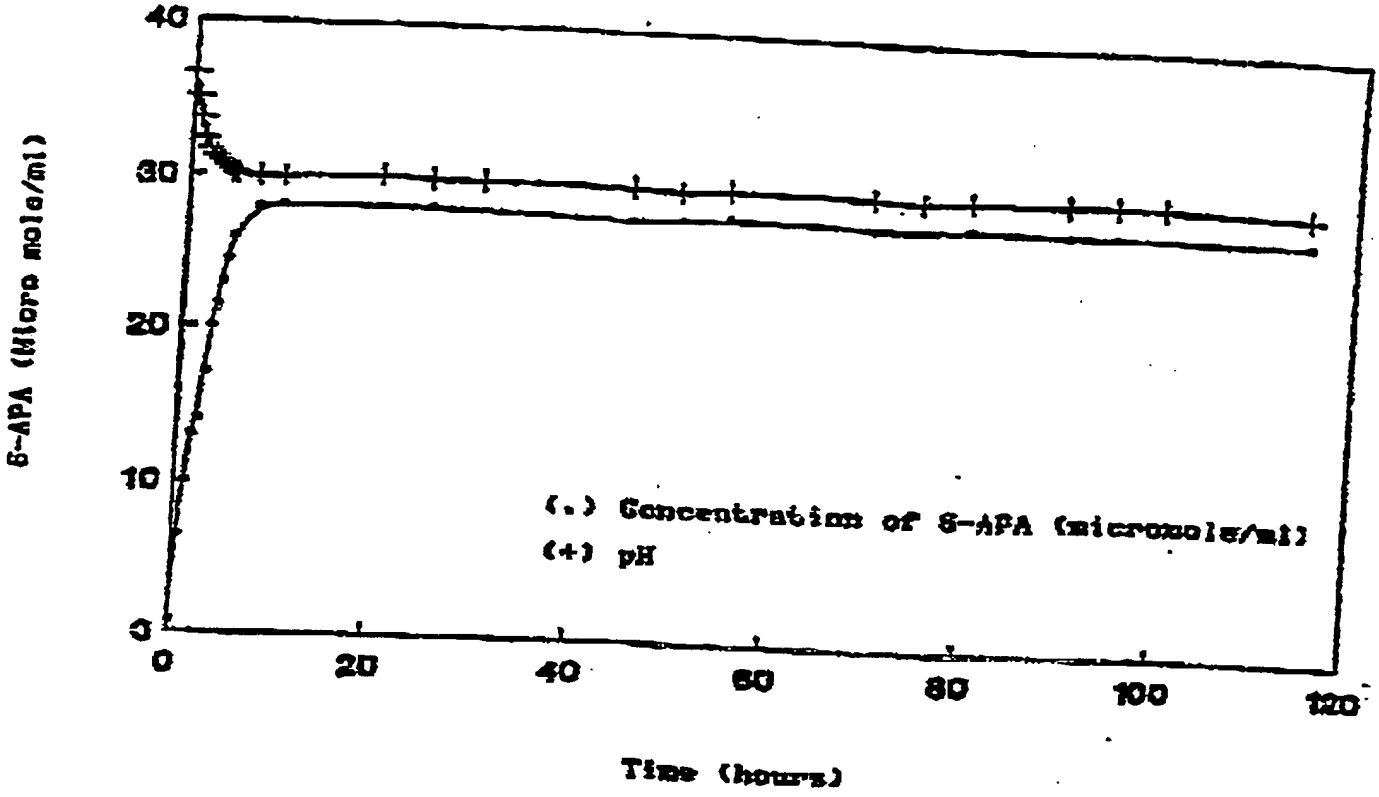

40
30
20
10
0
0
20
40
60
80
100
120
6-APA (Micro mole/ml)
(.) Concentration of 6-APA (micromole/ml)
(+) pH
Time (hours)

Fig. 4

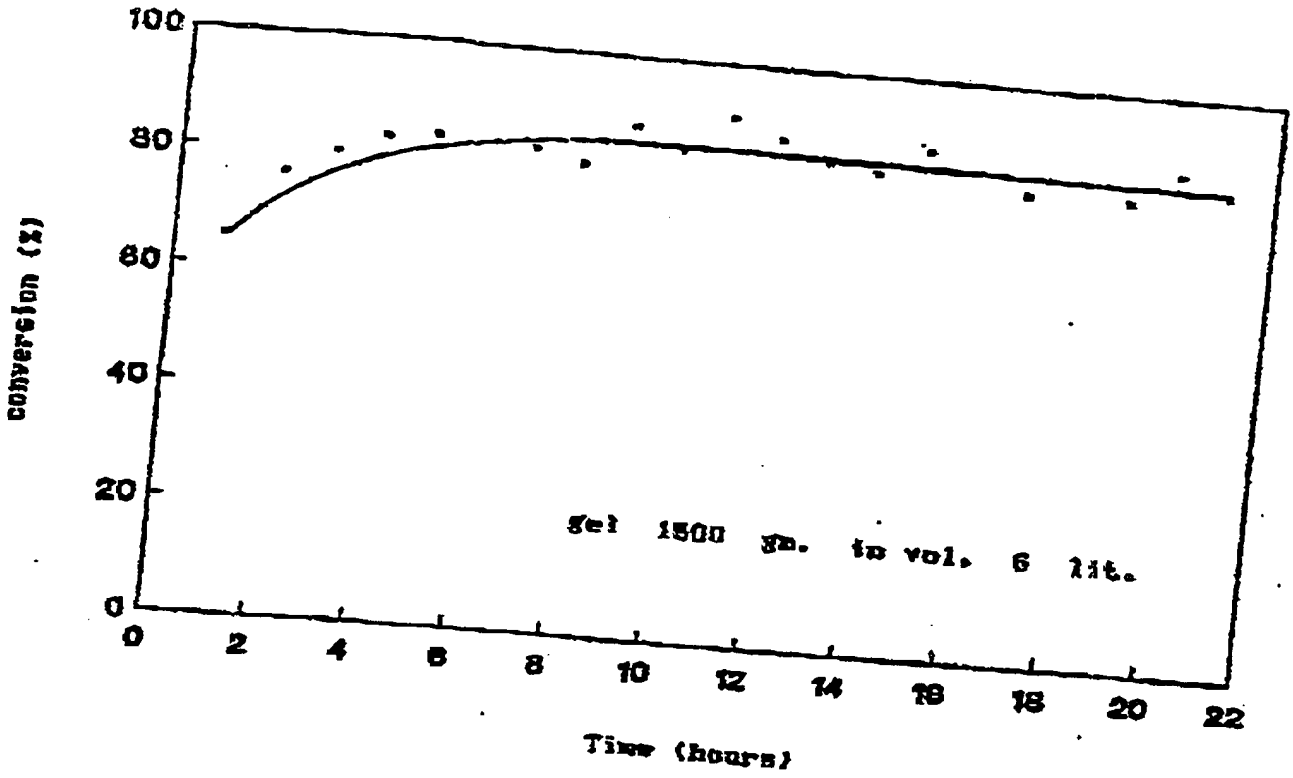

100
80
60
40
20
0
conversion (%)
Gel 1500 gm. in vol. 6 lit.
0
2
4
6
8
10
12
14
16
18
20
22
Time (hours)

Fig. 5

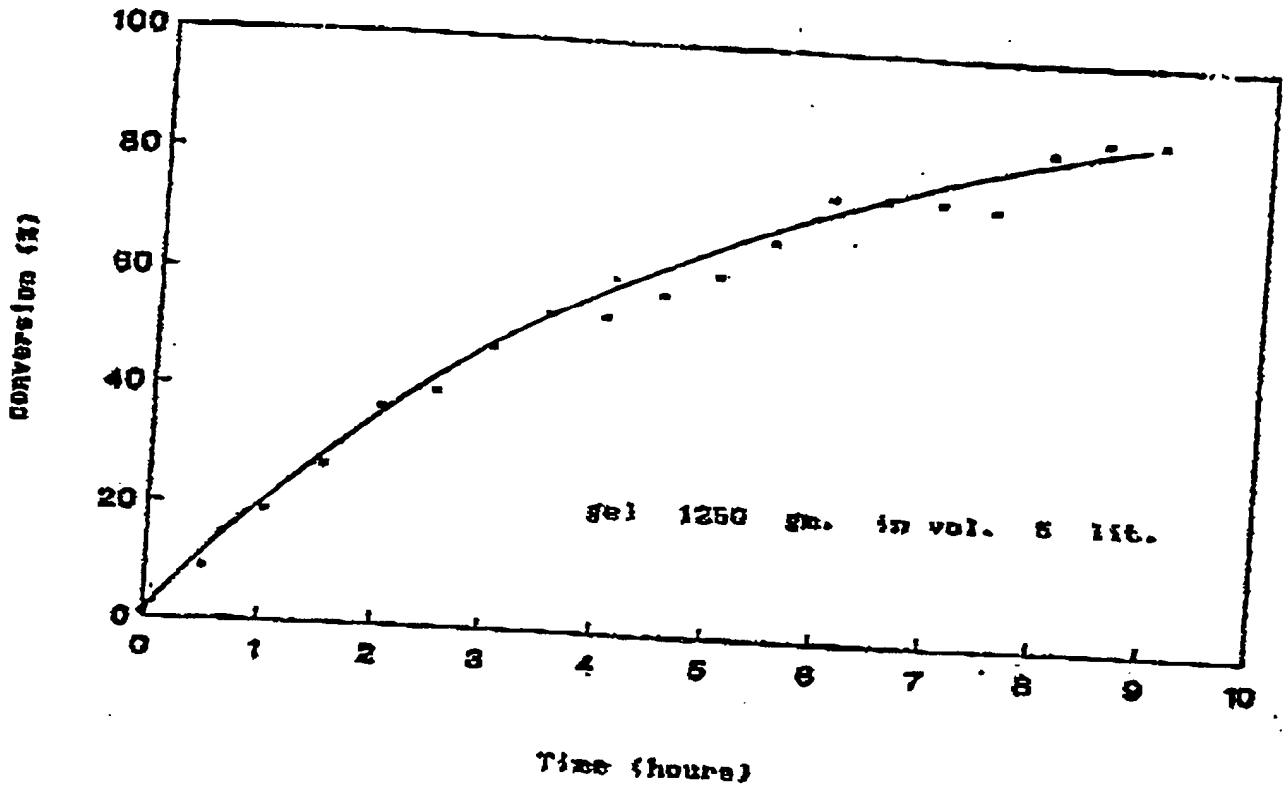

100
80
60
40
20
0
Conversion (%)
0
1
2
3
4
5
6
7
8
9
10
Time (hours)
Gel 1250 gm. in vol. 6 lit.

Fig. 6

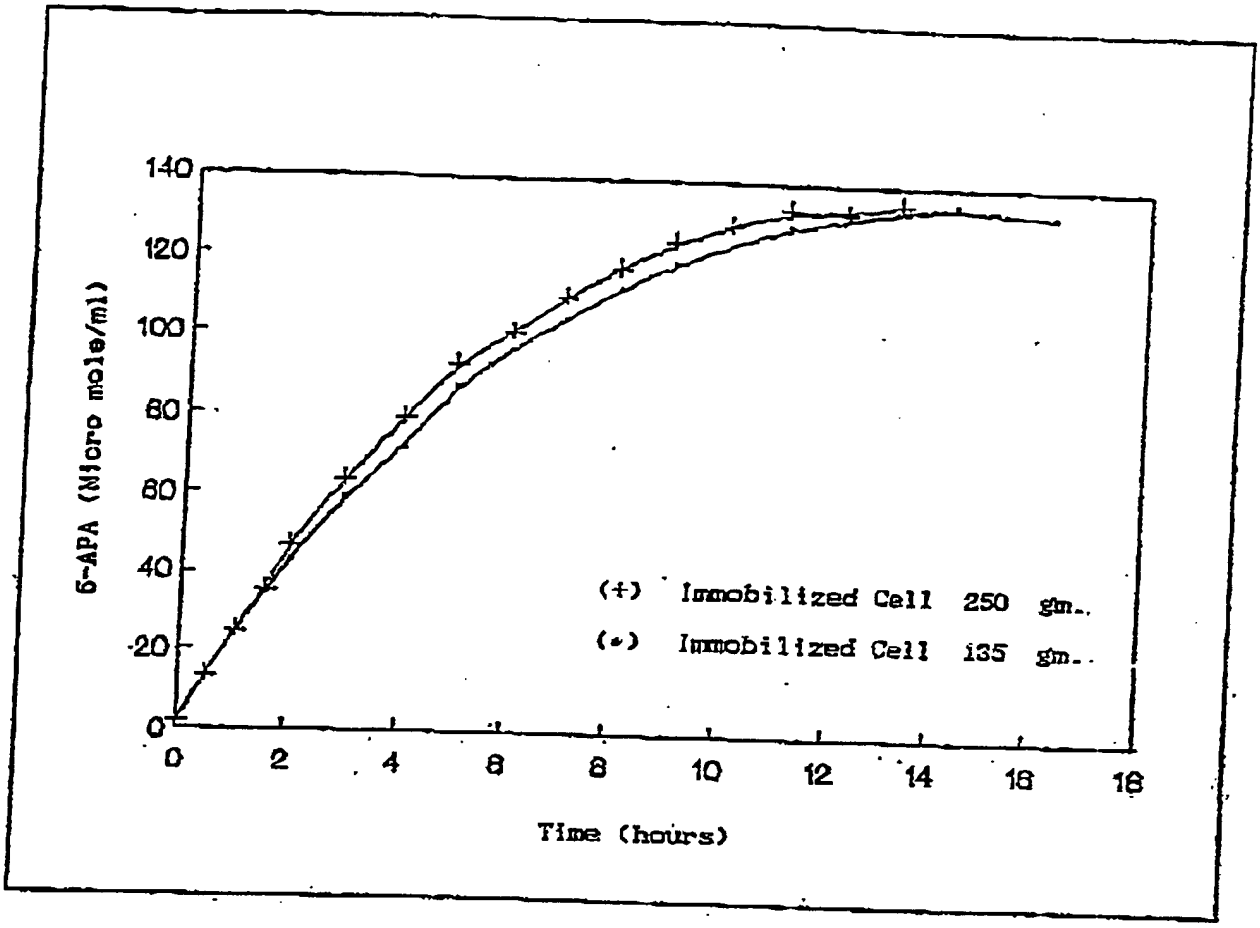

140
120
100
80
60
40
20
0
6-APA (Micro mole/ml)
0
2
4
6
8
10
12
14
16
18
Time (hours)
(+) Immobilized Cell 250 gm..
(•) Immobilized Cell 135 gm..

Fig. 7

IMMOBILIZED ENZYMES

This invention relates to immobilized enzymes, more particularly to immobilized acylases, and their use in de-acylation processes. The invention will be described with particular reference to the use of such enzymes to de-acylate antibiotics and related precursor materials having a fused beta-lactam structure including the penicillins. An important de-acylation process of the above kind is the preparation of 6-aminopenicillanic acid (hereinafter called "6-APA") which is an intermediate for the preparation of antibiotics of the penicillin group.

Background of the Invention

It is known that 6-aminopenicillanic acid can be prepared from precursors, such as penicillin G or penicillin V, by either of two methods one of which is a chemical process exemplified by US Patent No. 3,499,909. The chemical method has several disadvantages. The process, being a multistep process, requires a difficult and complicated control of the reaction, and the use of organic solvents for carrying out the reaction at a very low temperature (-40°C) under strictly anhydrous conditions, all of which results in a costly and energy-consuming process. The preferred alternative method employs the enzymatic action of penicillin acylase for hydrolyzing starting materials, such as penicillin G or penicillin V, to give the 6-APA product. This is a more convenient and more rapid process, since it is a single-step process which produces a purer product than the chemical process. At present, therefore, the enzymatic process is industrially more favoured. Examples of this process are US Patent Nos. 3,179,307; 3,260,653; 3,239,427 and 3,446,705.

The penicillin acylase used may be in the form of a crude enzyme isolated from cells or in the form of whole cells of certain micro-organisms. Several micro-organisms are capable of synthesizing penicillin acylase of high activity. Examples of such micro-organisms are Escherichia coli ATCC 9637, Escherichia coli ATCC 11105, Serratia rubidaea ATCC 181, Proteus rettgeri ATCC 9250, Bacillus megaterium ATCC 14945, etc.

Previously, the use of penicillin acylase in whole cells or cell-free extracts gave low yields of 6-APA. Products thus obtained were of low purity and the cost of production was still high since the enzyme could only be used once and was not recoverable. Moreover, the separation of the enzyme from the reaction mixture was not easy. The enzyme used was also of low stability.

To obviate the foregoing difficulties much effort has been made to develop satisfactory immobilized enzyme systems to obtain insoluble enzymes which can be recycled in the de-acylation process and which have improved stability. Processes for the preparation of immobilized enzymes are described in several patent specifications, for example, US Patent Nos. 3,536,587; 3,574,062; 3,607,653; 3,616,229; 3,619,371; 3,645,852; 3,650,900; 3,650,901; 3,925,157; 3,929,574; 3,957,580; British Patent Nos. 1,224,947; 1,274,869, German Patent Nos. 1,935,711; 2,012,089; etc.

Methods for immobilizing penicillin acylase in the form of enzymes and cells have been used for the production of 6-APA according to US Patent Nos. 3,925,157 and 3,953,291; German Patent No. 2,345,186.

Immobilizing processes may be classified into four categories, thus

(a) ionic binding on ion exchange resins or gels (Yamshof et al, "Biokhimiya", Atstr, Chemical. Abstr. Chemical Atstr. 46:9 (1981); Waxburton et al, Biotechnol Bioeng, 15:13).

(b) covalent binding by chemical linkage via functional group of the enzyme that are not essential to enzyme (German Offen., 2,215,512; 2,215,505; 2,215,687 and 2,215,539).

(c) immobilization by cross-linking the molecules of enzyme themselves into aggregate using bifunctional compounds (German Offen. 2,345,186), and

(d) entrappment or inclusion of the enzyme within a hydrophilic gel lattice which retains the enzyme but allows the substrate and product to pass through such as polyacrylamide, nitrocellulose (Chibata, I Enzyme Eng. 5:39 (1980); Shuydas et al, Bioeng. Khim, 3:546 (1977)).

Immobilized penicillin acylase is now widely used in processes for the industrial preparation of 6-APA (Warburton, D., et al. Biotechnol. Bioeng.; 15:13 (1980); US Patent No. 4,113,566). Production process may be of the fixed-bed reactor type (US Patent No. 3,953,291) or the stirred-tank reactor type which is the preferred type used in industry at present. The latter type requires the use of propeller within the reactor which results in the disintegration of immobilized enzyme possessing low stability (US patent No.3,957,580).

It has now been found that the use of certain gel entrappment materials, to be specified hereinafter, enables the preparation of immobilized enzyme systems of much improved properties, especially stability and strength. As a result the de-acylation process can be carried out at high efficiency under fluidized conditions and in a continuous manner.

The present invention comprises an immobilized material having enzymatic activity entrapped in a gel matrix, characterised in that the gel is of agarose, carrageenan, or a mixture thereof. The gel containing the enzymatic activity is preferably cross-linked e.g. with glutaraldehyde or glyoxal. Conveniently the immobilized material is in the form of beads. Other aspects of this invention are indicated in the appended claims.

The present invention also comprises an enzymatic deacylation process using immobilized material as described above, especially a process for the preparation of 6-APA by using immobilized penicillin acylase capable of hydrolyzing the starting material, which is penicillin G or penicillin V, to give 6-APA as a product. The acyl-group immobilized enzyme used is of high strength and stability, recyclable, and, if used in a continuous process, is of longer usable life, rapid catalytic action over wide ranges of pH and working temperature, conveniently controllable contamination, easy isolation of the reaction product. Employment of fluidization technique in the batch and/or continuous process enables the process to be smoothly, rapidly and efficiently carried out, producing high purity product.

The enzymatic process requires the use of immobilized penicillin acylase either isolated from the appropriate cells as crude enzyme or contained in the whole cells. Cells with high enzymatic activity are micro-organisms capable of synthesising enzyme at higher levels according to the US Patent Nos. 3,239,427 and 3,446,705, which disclose the use of available micro-organisms ie, Escherichia coli ATCC 9637, Escherichia coli, ATCC 1105, Proteus rettgeri ATCC 9250. Also disclosed is Proteus rettgeri SPS-6 which is a strain of bacteria having high activity of penicillin acylase. This strain can be cultured to produce enzyme in a minimum medium containing 0.4% of glucose.

The present process involves using entrappment by matrix substances such as agarose or carrageenan. One especially suitable substance is a mixture of agarose and carrageenan. Suitable conditions for immobilising cells of E. coli or Proteus rettgeri in agarose gel is 3 to 6% weight/volume (W/V), concentration of cell from 5 to 30% with an immobilising temperature of from 40 to 45°C. The cells are made into beads by a two-phase system (Nilsson et al Eur. J. Appl.Microbiol.; 17:319 (1983)). In the case where kappacarrageenan is used, the concentration of 2-5% (W/V), cell concentration of 5-30% at 40-45°C, and cell beads produced by a process according to Nillson et al (1983) are preferred conditions. It is unexpected that the use of a mixture of carrageenan and agarose produces higher strength of gel than normally obtained. The total mixture used is about 3-4% (W/V) of carageenan and agarose, and the preferred ratio of carrageenan:agarose is 2.0:10 or 2.5:1.5,etc. The cells are then immobilized by the method of Nilsson et al (1983). Gel beads obtained are then treated with a glutaraldehyde solution of 0.1 - 0.3 molar concentration, followed by treatment with hexamethylenediamine of 0.1 - 0.3 molar concentration. Yield of high strength gel beads is up to 0.7, 2.0 and 3.0 kilogram per square centimetre of agarose, carrageenan and mixture of carrageenan and agarose, respectively. Immobilized penicillin acylase obtained is of a very high stability, can be stored for one year without losing its enzymatic activity. Optimum conditions for the action of the three immobilized acylase enzymes are rather similar, ie, at a pH between 6.5 to 8.0, an optimum temperature for catalytic action of the enzymes is between 45 to 55°C and the range of pH-stability is 5 to 8.5.

The present process for preparing 6-APA from a water-soluble sodium or potassium salt of penicillin G or penicillin V comprises charging immobilized penicillin acylase into a fluidized-bed reactor. This technique has

hitherto not been used for the preparation of 6-APA. The technique is characterised by blowing air into a column containing immobilized enzyme in an appropriate amount to turn the gel beads into a fluid condition, wherein fluidization among 3 phases, ie. solid, liquid and gas, is possible (Figure 2 and Table 1). The advantage of fluidization is that the gel beads are constantly in motion, leading to rapid mixing and large contact area between gel beads and substrate. This method minimises energy consumption and dispenses with the control of flow of the substrate solution, as well as the product.

In the present process, a micro-organism cell concentration of from 10 to 30% (weight by weight) of the gel weight may be used. The immobilized penicillin acylase enzyme may be in any one of the following matrices: 5.0 - 6.0% of agarose, 2.5 - 5.0% of carrageenan or a mixture of carrageenan and agarose in a ratio of 2.5 to 1.5%. The immobilized cell beads have a strength of 1.0 to 5.0 x 10^2 kg/cm^2. When this high activity immobilized cells are used in the preparation of 6-aminopenicillanic acid from a water-soluble penicillin starting material in a bioreactor designed to operate under a fluidized condition in a temperature range of from 25 to 35°C and the pH is controlled to be within a range of 7.6 to 8.6. the yield of 6-aminopenicillanic acid is higher than 90%, regardless of whether the immobilized cells be of Escherichia coli ATCC 9637 or Proteus rettgeri ATCC 9250 or Proteus rettgeri SPS-6 (brownish red). The activity of penicillin acylase is between 25 to 30 units per gramme cells, 100 to 300 units per gramme gel (dry, weight). Immobilized enzymes of the present invention are suitable for use in the preparation process in a bioreactor, both of continuous and batch types. The substrate concentration used is 0.5 - 15% (W/V) of water-soluble salt of penicillin G or penicillin V. The immobilized enzyme can be stored at 4°C without any loss of enzymatic activity for 12 months, and at 25 - 30°C for 12 months with about 20% loss of activity. The catalytic

action of the enzyme is at its best in a pH range of from 7.5 to 9. Good hydrolysis of the substrate can still be obtained at a high temperature of 50 - 60°C.

The quantity of the immobilized enzyme used is in the range of 25-35% of the volume of the column of the bioreactor used for reaction. The pH must be adjusted to a constant value within the range of 7.6-8.6 with 10 molar ammonium hydroxide. Very small amount of heat is evolved during the reaction. Normally, a conversion rate of 90% and higher will be obtained if not more than 15% of a sodium or potassium salt of penicillin G or penicillin V is used.

Isolation by crystallisation of 6-aminopenicillanic acid obtained can be effected by lowering the pH to 4.2 (isoelectric pH) with 6N HCl or 6N H_2SO_4. Higher yields can be achieved by addition of methanol in a methanol:solution ratio of 1:1 or 1:2 which produces the maximum yield when the temperature is reduced to the level of 0-5°C. Methanol used is recoverable by distillation.

Immobilized penicillin acylase of the present invention can be used in the continuous process for the preparation of 6-aminopenicillanic acid for several months, or for at least 6 months in the batch process working at a period of 6 hours or less for each batch.

Example 1 Preparation of immobilized penicillin acylase from cells of *E. coli* ATCC 9637 in agarose gel.

In a 5-litre fermenter, culture *E. coli* ATCC 9637 in a minimum medium according to the formula of Self *et al* (*Biotech. Bioeng.*, 11:337 (1967)). Centrifuge the cells obtained at a velocity of 6500 x g for 15 minutes at 4°C. Wash the cells twice with a 0.85% sodium chloride solution (normal saline), then immobilise the cells in agarose.

Agarose powder is dissolved to give a solution of an appropriate concentration (3-5 g% agarose). Heat the solution at 116 ^{o}C for 10 minutes, then add 10-15% (W/V) of the prepared E. coli cells at 40 - 50^{o}C over a period of one minute. An equal volume of n-butyl acetate is added at 45^{o}C, agitation is continued until gel beads uniformly formed. Cool the mixture rapidly to 0-4^{o}C to give immobilized (enzyme) cells. Wash several times with cold water at a temperature not higher than 4oC. The cells obtained are stored in cold normal saline. immobilized penicillin acylase, contained in the cells of E. coli ATCC 9637, is reacted with 0-0.3 molar glutaraldehyde solution. Shake well at 20^{o}C for 2 hours, then the reaction mixture is shaken with 0-0.3 molar hexamethylenediamine at 4^{o}C for 30 minutes. The immobilized penicillin acylase is then washed 3 - 5 times with distilled water.

Example 2 Preparation of immobilized penicillin acylase from cells of E. coli ATCC 9637 in carrageenan.

Prepare cells of E. coli ATCC 9637 as in Example 1. The cells are then immobilized in K-carrageenan by the method of Example 1. The appropriate concentration of K-carrageenan is 2-5% weight by volume. Wash the immobilized penicillin acylase beads with 0.3 molar potassium chloride solution in place of cold water. Reactions with 0.1 molar glutaraldehyde and hexamethylenediamine are carried out in the same manner as in Example 1. Gel beads obtained are of twice the strength of the immobilized penicillin acylase prepared in agarose gel.

Example 3 Preparation of immobilized penicillin acylase from cells of E. coli ATCC 9637 in a mixture of carrageenan and agarose gel.

Prepare cells of E. coli ATCC 9637 as in Example 1. The cells are then immobilized in a mixture of K-carrageenan and agarose by the method of Examples 1 and 2. The ratio of K-carrageenan to agarose is 2.5:1.5 or 2:1% (W/V). Wash the gel beads obtained three times with 0.3 molar potassium chloride solution. Reactions with 0.1 molar glutaraldehyde and hexamethylenediamine are carried out in the same manner as above. Gel beads obtained are of twice the strength of that obtained in Example 2.

Figure 1 shows comparison of the strength of gel beads obtained from immobilization in agarose, K-carrageenan and a mixture of agarose and K-carrageenan, when the cells of E. coli ATCC 9637 of 10% (W/V) concentration are used.

Agarose gel	(5% W/V)
K-carrageenan	(2.5% W/V)
K-carrageenan/agarose	(2.5:1.5 W/V)

Apparatus and Experiment

Reactor is of a cylindrical form, with dimensions as shown in Table 1, made of Plexiglas or stainless steel. immobilized cells (enzyme) are contained within the reactor to act as catalyst for the conversion of penicillin G into 6-APA. Penicillin G solution is pumped by a diaphragm pump from a substrate tank and is fed through the bottom part of the reactor. This solution flows through the bed of immobilized cells (enzyme) which is in a fluidized condition by the compressed air fed through the bottommost part of the reactor . While flowing through the bed, the penicillin G solution is converted into 6-APA by the action of the immobilized enzyme in the beads. The solution then flows out through the top part of the reactor and is then

stored in a temperature-controlled tank so as to retain the activity of 6-APA. Air is let out through the topmost part of the fermenter as shown in Figure 2. Sampling valves are provided along the height of the reactor for periodical determination of the quantity of 6-APA produced.

Table 1: Details of fluidizing Reactor

Dimensions		1-litre (mm)	6-litre (mm)
Diameter of reactor	(A)	52	90
Diameter of head	(B)	99	150
Height of head	(C)	150	150
Height of cone	(D)	20	40
Height of reactor	(E)	600	775
Height of air feed	(F)	100	110
Material		Plexiglas or Stainless Steel	

Example 4 Rate of production of 6-APA in 1-litre fluidizing reactor in batch operation.

250 grammes of immobilized cells is charged into the reactor simultaneously with the addition of penicillin G solution of 8.0% by weight, in which the penicillin G is dissolved in a 0.05 molar solution of a phosphate buffer of pH 7.5 and 2.0% weight of KCl, to make 1 litre. Air is fed at an average rate of 1.35 litre per minute to create a

fluidized condition within the reactor. During the experiment, the pH of the reaction mixture is adjusted to remain within the range of 7.0-7.5 with 10 molar solution of ammonium hydroxide, and the temperatures are fixed at 25 ^{o}C and 30 ^{o}C . It has been found that at 30 ^{o}C, the production of 6-APA is 20 micromole/ml-hr, and at 25 ^{o}C the rate of production is 16 micromole/ml-hr. The results are shown in Fig. 3.

Example 5: Continuous production of 6-APA in 1-litre fluidizing reactor.

250 grammes of immobilized cells is charged into a 1-litre fluidizing reactor. A 2.0% by weight solution of penicillin G in 0.05 molar phosphate buffer solution of pH 7.5 and 2% by weight of KCl are continuously fed at a rate of 180 millilitres per hour, simultaneously with the feeding of air at a rate of 2.2 litres per minute. Allow the reaction to proceed continously without controlling the pH during the experiment which is carried out at room temperature. The experiment shows that the reaction enters an equilibrium state on the sixth hour and is capable of producing 6-APA at a concentration of 30 micromole/millilitre throughout the period of 120 hours. The state of equilibrium remains unchanged and the pH remains constant at 6.2 during the experiment, which demonstrates that the continuous production of 6-APA is efficient and the immobilized enzyme gives a high average yield as shown in Fig. 4.

Example 6: Continuous production of 6-APA in 6-litre fluidizing reactor.

1,500 grammes of immobilized cells is charged into a 6-litre fluidizing reactor. An 8.0% by weight solution of penicillin G in 0.05 molar phosphate buffer solution of pH

7.5 and 2% by weight of KCl are continuously fed at an average rate of 500 millilitres per hour, simultaneously with the feeding of air at an average rate of about 6.0 - 6.5 litres per minute. The conversion reaction of penicillin G to 6-APA continuously occurs while the pH is controlled at 7.0-7.5 with 10 molar solution of ammonium hydroxide. The temperature within the reactor is the same as the ambient temperature (ca. 32 ^{o}C). A 6-APA solution is obtained in a quantity of 147 micromole/millilitre, which is an equivalent of more than 85.0% conversion. The result of 22 days' experiment is shown in Fig. 5.

The 6-APA solution obtained is then cooled to 2-4 ^{o}C. n-Butyl acetate in an amount equal to a half of the quantity of the 6-APA solution is added with energetic stirring. The pH is adjusted to 1.5-2.0 with 25% HCl, then to 4.2 with 25% ammonia solution over a period of 3 hours. Filter and dry the product in vacuum for 3 hours.

With the fluidizing technique combined with continuous operation, the yield of 6-APA is as follows:

Recovery	58%
Purity of 6-APA	98%

Example 7: Rate of production of 6-APA in 6-litre fluidizing reactor in batch operation.

1,250 grammes of immobilized cells is charged into the reactor simultaneously with the addition of penicillin G solution of 6% by weight, in which the penicillin G is dissolved in a 0.05 molar solution of a phosphate buffer of pH 7.5 and 2% by weight of KCl, to make 6 litres. Air is fied at an average rate of about 6.0-6.5 litres per minute to create a fluidized condition. During the experiment, the pH of the reaction mixture is adjusted to remain within the range of 7.0 - 7.5 with 10 molar ammonium hydroxide

solution, and the reaction temperature is the room temperature. The concentration of 6-APA obtained is 150 micromole, which is an equivalent to a 90% conversion as shown in Fig. 6.

The 6-APA solution is then cooled to 2-4 ^{o}C. n-Butyl acetate in an amount equal to a half of the quantity of the 6-APA solution is added with energetic stirring. The pH is adjusted to 1.5 - 2.0 with 25% HCl, then the pH is brought to 4.2 with 25% ammonia solution over a period of 3 hours. Filter the white crystals of 6-APA obtained, and dry in vacuum for 3 hours.

With the fluidization technique and a batch operation, the results after crystallisation are as follows:

Recovery	61.0%
Purity of 6-APA	98.5%

Example 8: Comparison of the production of 6-APA in a 1-litre fluidized-bed reactor with the production of 6-APA in an enlarged 6-litre scale.

A study has been made to compare the production of 6-aminopenicillanic acid by a continuous process in a 1-litre fluidizing reactor with a similar production in a 6-litre apparatus, using immobilized cells under the following conditions:

Experimental conditions	Size of fluidized-bed reactor	
	1-litre	6-litre

Quantity of immobilized cells (gramme)	250	1,350
Concentration of penicillin G solution (%)	6	6
Rate of feeding of penicillin G solution (ml/hr)	40	220
Rate of feeding of air (litre/minute)	1.35	7.5
pH of solution	7.0-7.5	7.0-7.5
Temperature (^{o}C)	30	30

In this experiment, the yield from a continuous production of 6-aminopenicillinic acid through the use of immobilized cells in a 1-litre fluidized-bed reactor is compared with the yield from a similar production in a 6-litre reactor under the same experimental conditions, viz,. 6% by weight concentration of the penicillin G solution, pH of the reaction solution is adjusted to be within the range of 7.0-7.5, room temperature and the amounts of immobilized cells in the 1-litre and 6-litre reactors are 250 and 1,350 grammes, respectively. The feeding rates of penicillin G solution are 40 and 220 ml/hr for the 1-litre and 6-litre reactors, respectively. The feeding rates of air are 1.35 and 7.5 litre/minute for the 1-litre and 6-litre reactors respectively. It has been found that the maximum rates of reaction and yields of 6-aminopenicillanic acid produced in the two fluidized-bed reactors are equal. The maximum yields in both cases are 135 micromole per millilitre as shown in Fig. 7.

(Brief description of the drawings)

Figure 1: Influence of 0.1 molar glutaraldehyde solution and concentration of hexamethylenediamine on the activity of penicillin acylase and strength of gel beads:

(a) 10% (W/V) E. coli ATCC 9637 immobilized by 5% (W/V) of agarose (.);

(b) 10% (W/V) E. coli ATCC 9637 immobilized by 2.5% (W/V) of kappacarrageenan (*);

(c) 10% (W/V) E. coli ATCC 9637 immobilized by a mixture of kappacarrageenan and agarose in a ratio of 2.5:1.5% (W/V) (+).

Figure 2: Details and diagram of fluidizing reactor system.

Figure 3: Influence of temperature on the function of penicillin acylase enzyme contained in immobilized cells of Proteus rettgeri SPS-6 when used in the non-continuous production of 6-aminopenicillanic acid in a fluidized-bed reactor at 25 and 30 ^{o}C, at a penicillin G concentration of 8.0%, using 250 g of immobilized cells, rate of feeding of air is 1.35 l/min, pH controlled within the range of 7.0 - 7.5 with 10 molar ammonium hydroxide.

Figure 4: Continuous production of 6-aminopenicillanic acid in a 1-litre fluidized-bed reactor over 120 hours with 150 g of immobilized cells of Proteus rettgeri SPS-6, using 2.0% concentration of penicillin G, rate of feeding of penicillin G is 100 ml/hr, rate of air feeding is 2.2 l/min.

Figure 5: Influence of temperature on the function of penicillin acylase enzyme contained in immobilized cells of Proteus rettgeri SPS-6 when used in a non-continuous production of 6-aminopenicillanic acid in a fluidized bed reqactor at 25 and 30 ^{o}C, using 8% concentration of penicillin G, 250 g of immobilized cells, rate of air feeding is 1.35 l/min, pH is controlled within the range of 7.0-7.5 with 10 molar ammonium hydroxide.

Figure 6: Pattern of 6-aminopenicillanic acid production in 6-litre reactor under the conditions of Example 5.

Figure 7: Comparison between productions of 6-aminopenicillanic acid with immobilized cells of *Proteus rettgeri* SPS-6 in 1-litre and 6-litre reactors at room temperature. The concentration of penicillin G is 6.0%. In the 1-litre reactor (+), 250 g of immobilized cells is used and the rate of air feeding is 1.35 l/min, rate of feeding of penicillin G is 40 ml/hr. In the 6-litre reactor (,), 1,350 g of immobilized cells is used, rate of air feeding is 7.5 l/min, rate of feeding of penicillin G is 220 ml/hr. The pH is controlled within the range of 7.0 - 7.5 with 10 molar ammonium hydroxide.

CLAIMS

What we claim is:

1. An immobilized material having enzymatic activity entrapped in a gel matrix, characterised in that the gel is of agarose, carrageenan, or a mixture thereof.

2. An immobilized material according to claim 1, wherein the gel is cross-linked.

3. An immobilized material according to claim 2, wherein the gel is cross-linked with glutaraldehyde.

4. An immobilized material according to claim 1, 2, or 3, in the form of beads.

5. An immobilized material according to any of claims 1 to 4, wherein the gel contains from 3 to 6% (w/v) of agarose, or from 2.5 to 5% (w/v) of kappa carrageenan, or from 3 to 5% (w/v) of a mixture of carrageenan and agarose.

6. An immobilized material according to claim 5, containing a mixture of carrageenan and agarose in the proportion of from 1.5:1.5 to 3:2, preferably 2.0:1.0 or 2.5:1.5.

7. An immobilized material according to any of claims 1 to 6, having acylase activity, such as penicillin acylase activity.

8. An immobilized material according to any of the preceding claims, wherein the enzymatic material comprises whole cells.

9. An immobilized material according to claim 8, wherein the concentration of cells is from 5 to 30% (w/v)

10. An immobilized material according to any of claims 7 to 9, wherein the enzymatic material comprises cells selected from the group consisting of E.coli strains ATCC 9637 and ATCC 1105, Proteus rettgeri strains ATCC 9250 and SPS-6, and Bacillus megaterium strains including strain ATCC 14945.

11. An immobilized material according to any of claims 1 to 7, wherein the enzymatic material comprises a cell-free extract.

12. A process for immobilizing enzymatic material in a gel matrix which comprises entrapping the material in a gel of agarose, carrageenan, or a mixture threof.

13. A process according to claim 12, wherein after entrappment of the enzymatic material the gel is cross-linked with glutaraldehyede.

14. A process according to claim 13, wherein the gel is treated with 0.1-0.3 molar glutaraldehyde.

15. A process according to claim 13 or 14, wherein the cross-linked gel is treated with a diamine such as hexamethylene diamine.

16. A process according to claim 15, wherein the cross-linked gel is treated wilth 0.1-0.3 molar hexamethylene diamine.

17. An enzymatic de-acylation process characterised by the use of an immobilized material according to any of claims 1 to 11.

18. A process according to claim 17, in which the material is used in a fluidized condition.

19. A process according to claim 17 or 18, which is a continuous process.

20. A process according to claim 17, 18 or 19, for the preparation of 6-aminopenicillanic acid by treatment of a penicillin with a penicillin acylase.

21. A process according to claim 20, wherein the penicillin is a water-soluble form of penicillin G or penicillin V such as a sodium or potassium salt.

22. A bioreactor having a cylindrical shape, with a capacity of from 1 litre to 300 litres, wherein the diameter-to-height ratio is from 1:8 to 1:15, and comprising substrate feeding means at the lower part of said reactor, air inlet means for feeding air under pressure to the lowermost part of the reactor, the upper part of the reactor having an enlarged diameter (of about 1.6 times of the diameter of said reactor) and comprising outlet means for the product and air.

23. A process for the preparation of 6-aminopenicillanic acid, which comprises reacting immobilized penicillin acylase either as free penicillin acylase or cells having penicillin acylase activity, said immobilized penicillin acylase being in the form of beads prepared by a method according to any of claims 12 to 16, with a substrate containing a water-soluble salt of penicillin G or V, in a bioreactor according to Claim 22, the substrate being fluidized by air entering through an air distributor.

24. A process according to claim 23, wherein the column containing immobilized penicillin acylase has a capacity of from 1 to 300 litres.

25. A process according to claim 23 or 24, wherein the quantity of immobilized penicillin acylase used in the column of the fluidized-bed reactor is from 1/4 to 1/2 of the volume of the column used.

26. A process according to claim 23, 24, or 25, wherein the fluidizing air fed into said reactor column has a rate of flow of from 60 l/hr to 360 l/hr at a pressure of 1.0 kg/cm^2 and a temperature of from 30 to 40 ^{o}C.

27. A process according to any of claims 23 to 26, which is a batch process.

28. A process according to any of claims 23 to 26, which is a continuous process.

29. A process according to claim 28, wherein the solution of the penicillin compound is continuously fed at a feeding rate of from 80 to 25,000 ml/hr.

30. A process according to any of claims 23 to 29, wherein the reaction occurs at a temperature from 25 to 40 ^{o}C, a pH value of from 7.5 to 8.6, without accumulation of heat in the system when the cells of Proteus rettgeri and E. coli are used.

31. A process according to any of claims 23 to 30, wherein the quantity of said beads of immobilized penicillin acylase contained in said fluidized-bed reactor is from 1/4 to 1/2 of the volume of said column used.

32. A process according to any of claims 23 to 31, wherein the reaction is continuous or non-continuous in the same reactor.

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